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Three-dimensional structures of enzymes useful for β-lactam antibiotic production

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Significant advances have been made in the structure-based engineering of enzymes useful for β-lactam antibiotic production. Structure-based engineering of penicillin G acylase and cephalosporin acylase has resulted in improved enzymes for use in enzymatic production processes. The structures of many other enzymes that could be used in the production of β-lactam antibiotics, such as enzymes from the β-lactam biosynthetic pathway and β-lactam antibiotic-converting enzymes, have been determined. The interest in these structures suggests that the future may see an even more extensive use of rationally engineered biocatalysts in antibiotic production than today.

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Abbreviations

ACV	δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine
7-ADCA	7-amino-deacetoxycephalosporanic acid
AEH	α-amino acid ester hydrolase
6-APA	6-amino penicillanic acid
CA	cephalosporin acylase
CAH	cephalosporin acetyl hydrolase
CPC	cephalosporin C
DAOCS	deacetoxycephalosporin C synthase
DAOC/DACS	deacetoxycephalosporin/deacetylcephalosporin C synthase
GL-7-ACA	glutaryl-7ACA
IPNS	isopenicillin N synthase
PGA	penicillin G acylase

Introduction

Recent years have seen the elucidation of structures of several enzymes that catalyze the biosynthesis and bioconversion of β-lactam antibiotics, in the anticipation that structural information might help the rational engineering of these enzymes towards effective biocatalysts for the clean production of β-lactam antibiotics. In addition, the structural information could be used to alter an antibiotic-producing enzyme in such a way that it produces β-lactam antibiotics for which resistance is not (yet) an issue [1].

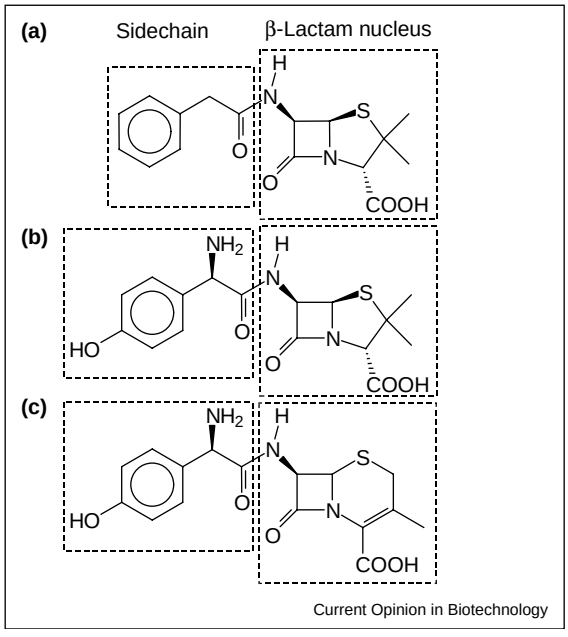
The β-lactam antibiotics can be described in terms of a β-lactam nucleus with a sidechain (Figure 1). Many different nuclei and sidechains are found in the antibiotics that are in use today. For instance, the naturally occurring penicillin G consists of the β-lactam nucleus 6-amino penicillanic acid (6-APA) to which a phenylacetic acid sidechain is coupled via an amide bond to the 6-amino group of 6-APA. Different combinations of sidechains and nuclei form antibiotics with distinctive properties; for example, replacing the phenylacetic acid sidechain of penicillin G with D-*p*-hydroxyphenylglycine results in the β-lactam antibiotic amoxicillin, which in contrast to penicillin G is orally stable. Likewise, the β-lactam nucleus can be varied. Combining the D-*p*-hydroxyphenylglycine sidechain of amoxicillin with the cephalosporin nucleus 7-amino-deacetoxycephalosporanic acid (7-ADCA) gives the cephalosporin cephadroxil. The numerous available sidechains and nuclei can thus be used in a multitude of combinations, each resulting in another β-lactam antibiotic.

Although the different sidechains are relatively easily synthesized on an industrial scale by chemical means, the chemical complexity of the β-lactam nucleus makes it impossible to synthesize these molecules economically in useful amounts. Instead, all β-lactam antibiotics produced today are derived from β-lactam nuclei obtained from the fermentation of various microorganisms. For example, the β-lactam antibiotic ampicillin is produced from penicillin G obtained from the fermentation of *Penicillium chrysogenum*: penicillin G is first hydrolyzed enzymatically to give the β-lactam nucleus 6-APA, which is then coupled to a D-phenylglycine sidechain to yield ampicillin. This coupling, as well as the coupling to other sidechains, can be done chemically, but enzymatic methods are being developed with the help of structural research reviewed below (also see Figure 2). Similarly, other nuclei — like those of the cephalosporins — can be obtained from fermentative processes and enzymatic cleavage of the sidechain.

Enzymes for sidechain conversions

Penicillin acylases are used in industry to catalyze the hydrolysis of the naturally occurring penicillins G and V to produce the β-lactam nucleus 6-APA, which is then chemically converted into other antibiotics. Two different enzymes — penicillin G acylase (PGA) and penicillin

Figure 1



β-Lactam antibiotics comprise a β-lactam nucleus coupled to a sidechain. Examples of two penicillins **(a)** penicillin G and **(b)** amoxicillin and **(c)** a cephalosporin, cephadroxil.

V acylase (PVA) — are currently used, of which PGA from *Escherichia coli* is the most important. Together, these enzymes are responsible for the production of thousands of tons of 6-APA annually [2].

PGA (Figure 3) is a member of the Ntn hydrolase family, members of which share a typical fold of a double layer of β sheets, sandwiched between α helices. The enzymes are produced as proenzymes, which are self-activated by the autocatalytic cleavage of the polypeptide resulting in an α and a β chain. The resulting N-terminal residue of the β-chain is always a serine, threonine or cysteine which acts as the nucleophile in both the maturation reaction and the hydrolysis of the substrate [3]. The nucleophilicity of the hydroxyl or thiol group of this residue is enhanced through general base catalysis by its own N-terminal amino moiety.

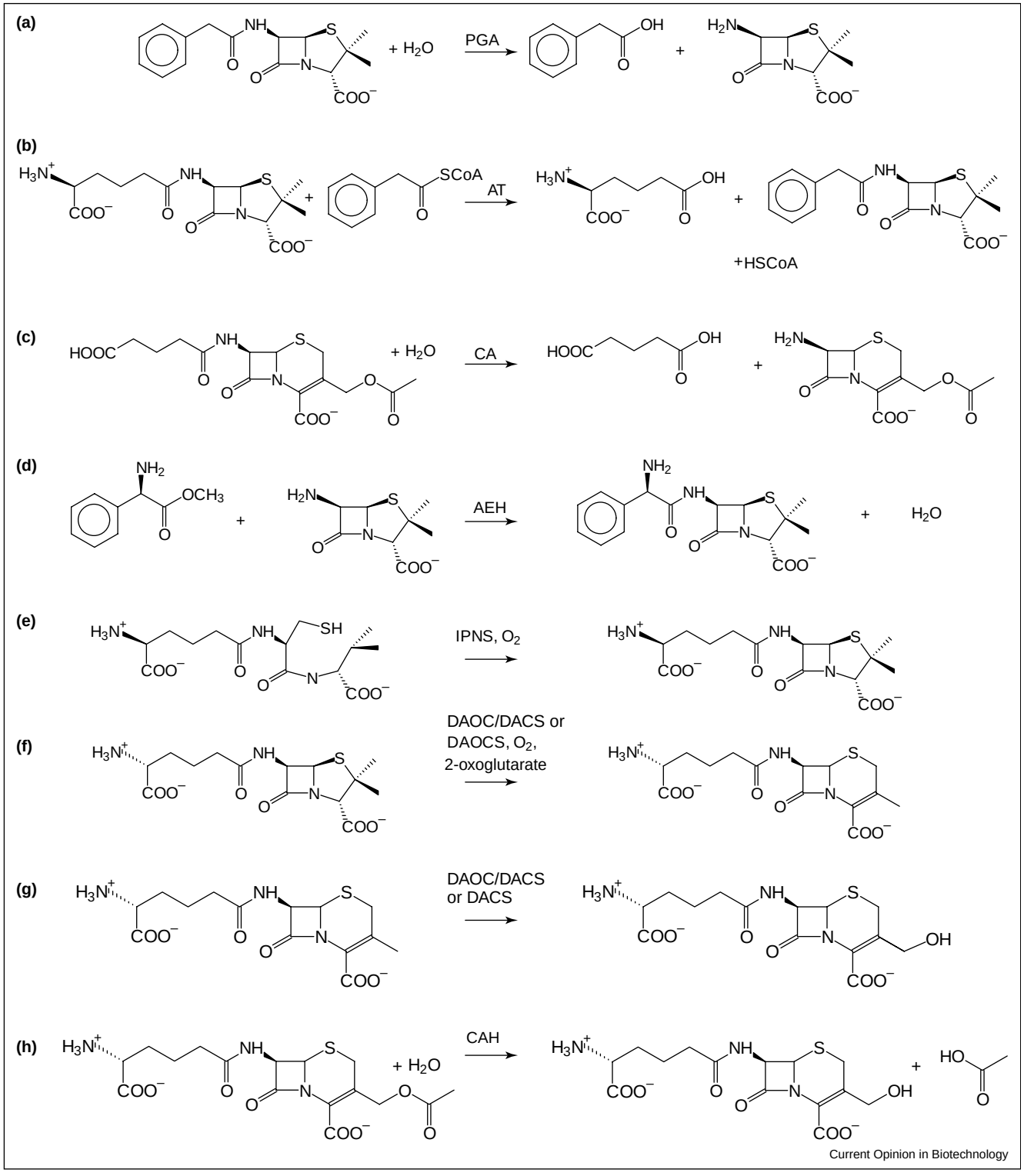
In the PGA-catalyzed hydrolysis of penicillin G (Figure 2a), the nucleophilic serine attacks the carbonyl carbon atom of the amide bond between the phenylacetic acid sidechain and the 6-APA nucleus, liberating 6-APA and forming an acyl enzyme. The acyl enzyme is then hydrolyzed, resulting in phenylacetic acid and a regenerated active site. The specificity of PGA for the phenylacetyl sidechain, and the localization of its gene close to genes encoding enzymes involved in *p*-hydroxyphenylacetic acid degradation, suggest that *in vivo* the enzyme provides a means for the conversion of phenylacetylated compounds [4]. Three-dimensional structures of the enzyme show a clear binding pocket for a phenylacetyl group, but the enzyme can bind other compounds too, responding through conformational changes involving sidechain motions, notably of αArg145 and αPhe146 [4]. PGA can also be used for the coupling of sidechains to an amino-β-lactam. To this end, the enzyme is acylated by an ester or amide of the desired acyl sidechain and the resulting acyl enzyme is 'aminolysed' by an amino-β-lactam, resulting in a semisynthetic antibiotic. As the acyl enzyme can also react with a water molecule and be hydrolyzed, and because the antibiotic product may also be hydrolyzed by the enzyme, ways are being sought to diminish these undesired hydrolytic side reactions and so to increase the yield [5].

The rational design of PGA mutants with such increased production levels requires knowledge of the way in which amino-β-lactams and the antibiotic products bind to the acyl enzyme. Unfortunately, a clearly defined binding pocket for the β-lactam nucleus cannot be distinguished in the crystal structure of wild-type PGA. However, crystal structures of an inactive PGA mutant in complex with penicillin G (Protein Data Bank [PDB] codes 1FXV [5] and 1GM7 [6]) and of the wild-type PGA in complex with the slowly hydrolyzed penicillin G sulfoxide (PDB code 1GM9 [6]) have been determined and resulted in several residues being implicated in penicillin binding. Mutagenesis of active-site residues resulted in mutant PGAs with an increased propensity to catalyze antibiotic synthesis rather than the hydrolytic side reactions [5,7,8]. This research also led to the design of three mutants (αPhe146Tyr, βPhe24Ala and αPhe146Tyr/βPhe24Ala) that have increased affinities for Cα-substituted phenylacetic acid derivatives, making them interesting for the coupling of such compounds to β-lactams. Crystal structures suggested that van der Waals interactions with the phenyl hydroxyl group introduced by the αPhe146Tyr mutation explain the increased affinity for α-substituents on the substrate (WBL Alkema *et al.*, unpublished).

Acyl coenzyme A: isopenicillin N acyltransferase (AT) is found in the biosynthetic route towards penicillin G, where it catalyzes the exchange of the α-amino adipic acid sidechain of isopenicillin N for phenylacetic acid, resulting in penicillin G (Figure 2b). As this enzyme accepts a wide variety of sidechains, it might become a very useful tool in antibiotic production and structural studies are under way [9].

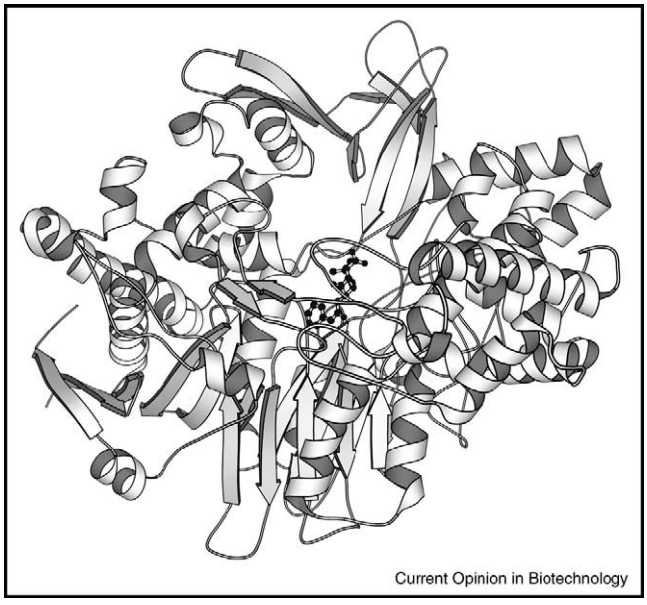
Cephalosporin acylase (CA; also called glutaryl acylase) can be used in enzymatic production methods for semisynthetic cephalosporins. Starting from fermented cephalosporin C (CPC), the cephalosporin nucleus 7-amino cephalosporanic acid (7-ACA) is commonly obtained

Figure 2



Reactions catalyzed by the enzymes reviewed in the text. **(a)** Penicillin G hydrolysis by penicillin G acylase (PGA). **(b)** Isopenicillin N conversion to penicillin G by acyl-coenzyme A: isopenicillin N acyltransferase (AT). **(c)** Glutaryl 7-ACA hydrolysis catalyzed by cephalosporin acylase (CA). **(d)** Acylation of 6-APA with phenylglycine methylester catalyzed by α-amino acid ester hydrolase (AEH). **(e)** Formation of isopenicillin N from the tripeptide ACV by isopenicillin N synthase (IPNS). **(f)** Ring expansion of isopenicillin N into deacetoxycephalosporin C by deacetoxycephalosporin C synthase (DAOCS). **(g)** Hydroxylation of deacetoxycephalosporin C by deacetoxycephalosporin/deacetylcephalosporin C synthase (DAOC/DACS). **(h)** Deacetylation of cephalosporin C by cephalosporin acetyl hydrolase (CAH).

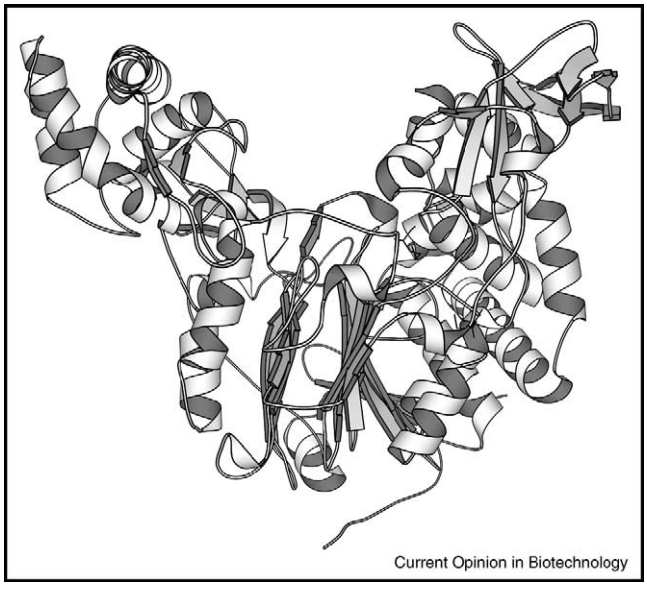
Figure 3



The fold of *Escherichia coli* penicillin acylase. This figure was prepared from the coordinates of the inactive mutant complexed with penicillin [5]. Penicillin is shown in ball-and-stick representation. (Figure drawn with Molscript [27].)

using hazardous, noxious chemicals. A more environmentally friendly alternative is offered by enzymatic hydrolysis using CA. However, the catalytic activity of CA on CPC is quite low compared to its activity on the natural substrate glutaryl-7ACA (GL-7-ACA) shown in Figure 2c. Therefore, the enzyme is being engineered to increase its activity on CPC [10].

Figure 4



The crystal structure of *Pseudomonas diminuta* cephalosporin acylase [11]. (Figure drawn with Molscript [27].)

Kim and colleagues determined the crystal structures of both the inactive precursor and the active, mature CA of *Pseudomonas diminuta* (PDB codes 1KEH and 1FM2 [11,12]; Figure 4) The core of the enzyme showed an Ntn hydrolase fold, and the active site was found to be similar to that of penicillin acylase, although CA and PGA show a very different structure away from the active site [12]. Structures of CA with bound GL-7ACA and glutarate (PDB codes 1JBZ and 1JWO, respectively) identified the residues that are important for substrate binding (αTyr149, αArg155, βLeu24, βTyr33, βGln50, βArg57, βPhe177, βVal70, βSer1 and αSer152 [10]). In both complexes, the glutaryl sidechain is buried inside a deep pocket, and the observed interactions in the active site suggest an explanation for the enzyme's preference for GL-7-ACA over CPC. Because the D-α-aminoadipyl sidechain of CPC is larger than the glutaryl sidechain in GL-7-ACA, it would collide with the binding site residues. Therefore, Kim and Hol [10] proposed to mutate bulky residues in the binding site to smaller ones to increase the activity on CPC. From structures of CA with bound phosphate and ethylene glycol and with bound glycerol, Fritz-Wolf *et al.* [13] also proposed mutations that should allow CA to act more efficiently on CPC. Site-directed mutagenesis based on the substrate-binding mode observed by Kim and Hol [10] eventually resulted in a triple mutant (βGln50Met/αTyr149Lys/βPhe177Gly) that exhibited drastically increased deacylation activity on CPC — up to 790% that of the wild type [14•].

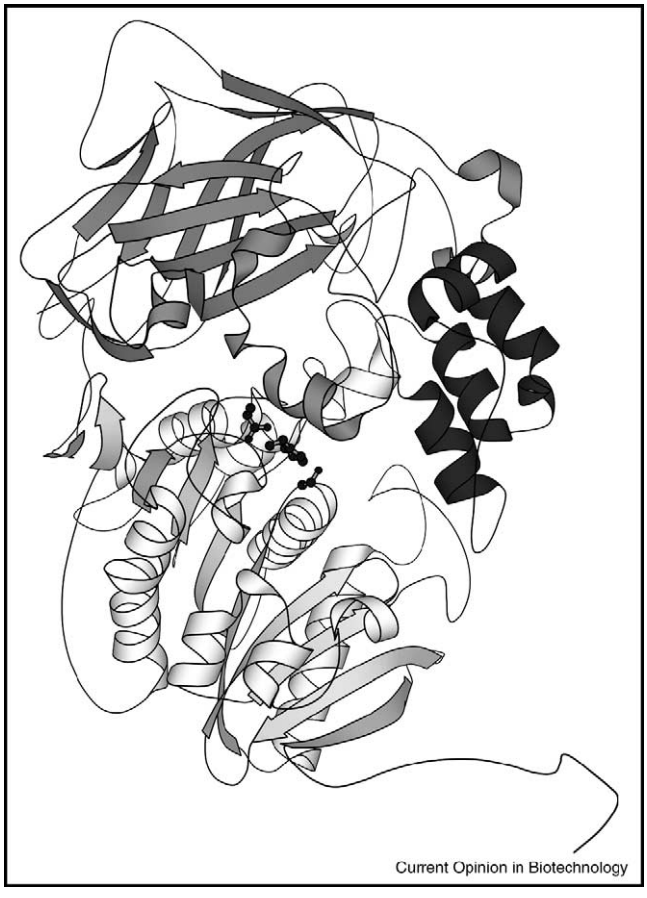
Enzyme structures may also aid the understanding of directed evolution studies. Otten *et al.* [15] have evolved *Pseudomonas* SY-77 CA into a more efficient adipyl acylase for the conversion of adipyl-7-ADCA into the free cephalosporin nucleus 7-ADCA by the selection of clones from a library of mutants. The best enzymes showed mutations in positions close to the substrate-binding site. Strikingly, in this study, residue Asn266 was implicated in the substrate specificity for the first time, and the Asn266His mutant showed increased activity on both glutaryl- and adipyl-7-ADCA. A model based on the crystal structure of the CA enzyme in complex with glutaryl-7-ACA suggested that Arg255 is responsible for the binding of the glutaryl sidechain's carboxylate group. The Arg255 sidechain is kept in the correct position for this interaction by hydrogen bonds involving Asn266. Mutating Asn266 was proposed to perturb these interactions and thus alter the substrate selectivity.

Like penicillin acylases, the α-amino acid ester hydrolases or AEHs are capable of catalyzing the removal and attachment of the sidechain of β-lactam antibiotics. Importantly, however, the AEHs have a very strong

preference for substrates with an α-amino group on the acyl chain. Thus, they can be used to synthesize important antibiotics like ampicillin, amoxicillin, cephalexin and cephadroxil, which have either a D-phenylglycine or a *p*-hydroxy-D-phenylglycine sidechain. As an example, Figure 2d shows the AEH-catalyzed synthesis of ampicillin.

Unlike PGA, the AEHs are not inhibited by phenylacetic acid and can therefore be very useful in a one-pot transacylation scheme [16], in which a natural antibiotic, an ester (or amide) of the desired new α-amino acyl chain, a PGA and an AEH are present at the same time. The PGA can catalyze the deacylation of penicillin G, while the AEH acylates the resulting 6-APA by reaction with the new acyl donor. Although the first AEHs were purified in the 1980s, the first AEH genes were cloned only in the past few years [17,18] so that rational engineering has only recently become a possibility.

Figure 5



The fold of *Xanthomonas citri* AEH [18]. The different domains are individually shaded: the α/β-hydrolase fold domain is in light gray; the jellyroll fold domain in medium gray; and the cap domain in dark gray. The residues of the catalytic triad are indicated in ball-and-stick representation. (Figure drawn with Molscript [27].)

Sequence alignments, mutational analyses and labeling experiments suggested that the AEHs are α/β-hydrolase fold enzymes employing a classical Ser-His-Asp catalytic triad [17,19]. This was subsequently confirmed by the crystal structure of the *Xanthomonas citri* AEH [18] (PDB code 1MPX). The structure (Figure 5) showed a three-domain fold including an α/β-hydrolase domain, a predominantly helical cap domain and a domain with a jellyroll fold. The active site is found at the interface of the three domains, with the catalytic triad residues Ser174, His340 and Asp307 located in their canonical positions in the α/β-hydrolase domain. A putative binding pocket for the acyl chain is formed by residues from the α/β-hydrolase and cap domains. Strikingly, a cluster of three acidic residues (Glu309, Asp310 and Asp208) protrudes into this pocket forming a well-defined interaction site for the substrate's amino group, explaining the substrate specificity. As with PGA, however, the native structure showed no clear binding site for a β-lactam acyl acceptor. Modeling tentatively suggests the involvement of Tyr82, which is also involved in catalysis, as one of the oxyanion-stabilizing residues.

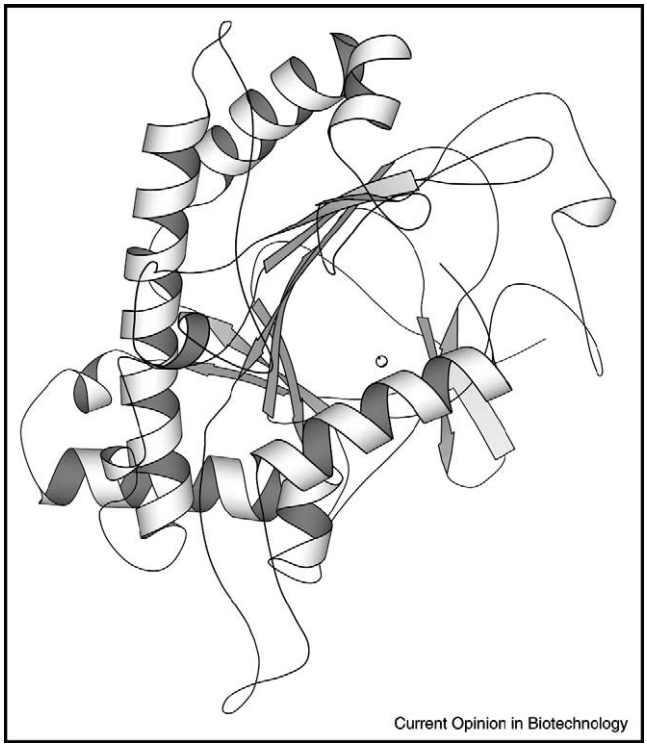
Structural studies on AEHs could, as was the case for PGA, help the design of mutants with improved biocatalytic properties or even of mutants able to accept completely new sidechains for the production of new antibiotics. To this end, residues on the cap domain, like Trp209, Tyr222 and Asn219, which seem to be involved in recognition of the acyl chain, may be mutated. Alternatively, the carboxylate cluster may be altered with a view to drastically changing the enzyme's specificity

Enzymes for β-lactam nucleus formation

Isopenicillin N synthase (IPNS) [20] and deacetoxycephalosporin C synthase (DAOCS) [21] are enzymes involved in the biosynthesis of the β-lactam nucleus that are structurally well characterized. In a review in 2001, Andersson *et al.* [1] proposed that such enzymes could be modified to accept new substrates, allowing the formation of intermediates that are more easily used in subsequent steps of production processes or even enabling the direct formation of compounds that do not require any additional conversions but are immediately ready for use as a drug.

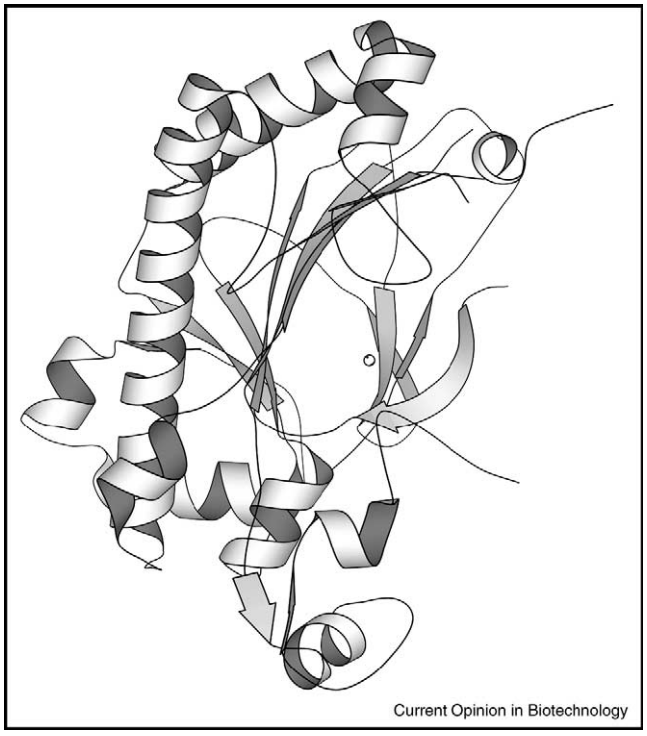
IPNS and DAOCS share a common structural scaffold with a central jellyroll fold surrounded by α helices (Figures 6 and 7) and both use a catalytic mechanism based on a ferrous iron atom [1]. IPNS occurs in the biosynthetic route for penicillin G formation where, without assistance, it synthesizes the entire intricate two-ring β-lactam structure of the penicillin precursor isopenicillin N from the tripeptide δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine (ACV). This process is an oxidative reaction fueled by molecular oxygen (Figure 2e). Working under anaerobic conditions to stop the enzyme in its tracks,

Figure 6



The structure of IPNS [20]. The catalytic iron atom is shown as a sphere. (Figure drawn with Molscript [27].)

Figure 7



Fold of DAOCS [21]. The catalytic iron atom is shown as a sphere. (Figure drawn with Molscript [27].)

Roach *et al.* [20] have obtained the crystal structures of IPNS in complex with ACV and iron and of IPNS in complex with iron, ACV and nitric oxide, which is a non-reactive analog of oxygen. The high resolution of their diffraction data (1.3 Å and 1.45 Å, respectively; PDB codes 1BK0 and 1BLZ) allowed a detailed view of the active site. This showed a square-pyramidal coordination of the iron with the cysteinyl-derived sulfur atom of ACV coordinating the iron apically. Nitric oxide binds laterally, in a nonlinear way (i.e. with an obtuse angle between Fe, N and O), which allowed the proposal of a reaction mechanism. If oxygen also binds in such a nonlinear way, it would be perfectly placed to abstract two hydrogen atoms from ACV, resulting in β-lactam ring closure and the formation of one water molecule from one of the oxygen atoms on the iron. The remaining oxygen could then abstract a hydrogen atom from the valine sidechain of ACV, inducing attack on the sulfur atom and formation of the second (thiazolidine) ring. A later structural study [22] (PDB codes 1QJF, 1QIQ and 1QJE) supported this sequence of events by trapping a monocyclic intermediate in the crystal using a specially selected ligand that did convert into a monocylic β-lactam, but which did not form the thiazolidine ring. Long *et al.* [23] have reported structures of IPNS in complex with a truncated substrate (PDB codes 1OC1 and 1OBN), which is converted into three different products. Combining these structures with modeling studies, they investigated the product selectivity of IPNS acting on substrates other than ACV. This may provide useful information for the engineering of IPNS for the development of new synthetic procedures.

The bacterial enzyme DAOCS catalyzes a ring expansion, turning the five-membered thiazolidine ring of a penicillin nucleus into the six-membered dihydrothiazine ring of a cephalosporin (Figure 2f), and has a central role in the biosynthesis of cephalosporins by prokaryotes [21]. This ring expansion is also an oxidative process requiring molecular oxygen but, in contrast to IPNS, DAOCS also requires 2-oxoglutarate, which is used in the oxidative half reaction needed to generate the iron (IV) center. In this half reaction, the oxoacid is decarboxylated to generate carbon dioxide and succinate.

The structures of DAOCS from *Streptomyces clavuligerus* alone and with bound 2-oxoglutarate were solved by Valegård *et al.* [21] (PDB codes 1RXF and 1RXG). Recently, Valegård *et al.* [24••] have explained how the extremely unstable iron (IV) in DAOCS is kept in check until its reactivity is required. By elucidating several crystal structures of enzyme–substrate and enzyme–product complexes (PDB codes 1UO9, 1UOF, 1UOB, 1UNB and 1UOG), they show that the binding sites for the 2-oxoacid and the β-lactam overlap. They propose that after

the generation of the iron (IV) species through oxidation by oxygen and carbon dioxide expulsion from the 2-oxoglutarate, the succinate product remains bound to the iron and stabilizes its charge. The penicillin substrate, competing for the same binding site, displaces the succinate and in doing so causes oxidative attack on itself by the iron, which the authors refer to as being 'booby-trapped' in this way. The paper by Valegård *et al.* continues to propose a detailed reaction mechanism for ring expansion in which the iron (IV) binds the sulfur atom of the thiazolidine ring. An oxygen atom bound to the iron abstracts a hydrogen atom from one of the thiazolidine ring's methyl groups, generating a radical on this methyl group. This then attacks the sulfur atom, resulting in its inclusion in the ring and deacetoxycephalosporin C formation.

Thus, crystal structures have served to elucidate the mechanism of β-lactam biosynthesis. Moreover, the resulting structures show the precise interactions between β-lactam-forming and β-lactam-converting enzymes and their substrates, which makes rational engineering a possibility.

Two other enzymes, DACS and DAOC/DACS, are also involved in cephalosporin biosynthesis. DACS hydroxylates deacetoxycephems, whereas DAOC/DACS shows both DACS and DAOCS activity (Figure 2f,g). Although no structures are available of either DACS or DAOC/DACS, the structural information on DAOCS can be extrapolated to these enzymes. Recently, Lloyd *et al.* [25••] have used homology modeling of DAOC/DACS and DACS based on the *S. clavuligerus* DAOCS structure to identify important residues in DAOC/DACS from *Cephalosporium acremonium*, which shows 57% sequence identity to the *Streptomyces* DAOCS. It was shown that single amino acid replacements could strongly increase one of the activities of DAOC/DACS relative to the other, but the molecular details of this remarkable success remain to be elucidated.

Figure 8

Fold of a monomer of the hexameric cephalosporin deacetylase from *Bacillus subtilis* [26]. (Figure drawn with Molscript [27].)

A completely different class of enzymes, the so-called cephalosporin acetyl hydrolases, might also be of use in the enzymatic production of antibiotics. Vincent *et al.* [26] have reported the structure (Figure 8) of cephalosporin acetyl hydrolase (CAH) from *Bacillus subtilis* 168 (PDB code 1ODS), which can remove the acetyl group from the C10 position on the cephalosporin C nucleus (Figure 2h). This enzyme is an α/β-hydrolase with a Ser-His-Asp catalytic triad. It might be possible in the future to engineer the structure of this enzyme to enable the (de)acylation of cephalosporins on the C10 position with various acyl chains, which may open up a route to new antibiotics [26].

Conclusions

A large number of enzymes is being investigated with a view to engineering them for use in the industrial production of β-lactam antibiotics. Given this interest, in the near future enzymatic semisynthesis may replace chemical methods for antibiotic production, playing an even greater role in their manufacture than at present. Structural research has contributed much to this ongoing development by yielding the structures of enzymes useful for both sidechain and β-lactam nucleus conversions. The examples of penicillin G acylase and cephalosporin acylase, for which several mutants with improved properties have already been prepared with the aid of structural research, hold promise for the engineering of the other enzymes reviewed in this paper.

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